High-density Cultivation in the Production of Microbial Products

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doi: 10.15255/CABEQ.2018.1394

Review
Received: May 31, 2018
Accepted: December 28, 2018

Microbial fermentation processes are of great importance for the production of many bioproducts. Even for established processes, improvements in product yield, productivity, and quality are always continually demanded. This is particularly true as the products mature from being novelty to high demand, even bulk, substances, as has been witnessed for several antimicrobial compounds. High-density cultivations have been found very useful in producing a large number of modern bioproducts. Selection of the mode of fermentation, operating conditions, and optimized media are important characteristic features for high cell density, productivity, as well as the commercial success of any microbial product. This contribution reviews some of the challenges and technologies investigated for high-density cultivation. Important aspects such as medium composition, reactor conditions, oxygen transfer, temperature, agitation, pH, modes of operation, and feeding strategies for high-density cultivation are summarized in this review.

Keywords:

high-density cultivation, mode of fermentation, medium composition, reactor conditions, biomass

Introduction

Large-scale culturing of microorganisms possessing biochemical pathways for desired products has enabled production of compounds such as enzymes, alcohols, amino acids, antibiotics, organic acids, and other biologically active compounds in quantities that might otherwise have been very difficult to obtain from natural sources or by chemical synthesis. Lately, new constructs of microbial production strains are being devised to even secrete the recombinant proteins[1]. As the products mature, process economics demands increasing volumetric productivity (i.e., production per unit volume per unit time; a product of active cell density and the specific cell productivity), which is a major goal of modern bioprocesses[2–4]. While metabolic engineering and regulation of biochemical pathways are utilized to increase specific cell productivity[5–7], high-density cultivations are designed to achieve high product concentrations in broth via growing cells to high densities while maintaining high specific cell productivity. As a result, high-density cultivation (HDC) has become an important tool in modern bioprocessing. This review concerns with the different issues involved in HDC of microorganisms.

High cell density is a relative term and no exact concentration of dry cell weight is considered representative of the process. In general, cell concentrations an order of magnitude or more than those achieved in conventional cultivation processes have been considered as high cell densities[8]. Per Tapia *et al.*[8], cell concentration in the order of 10^7 cells mL^{-1} is representative of high cell density. On the other hand, other studies have used dry cell weight densities ranging from 20 g L^{-1} [9] to 50 g L^{-1} [10], and as high as to 200 g L^{-1} [11] and 300 g L^{-1} [12], to designate a high-density cultivation.

In the same note, although HDC research was initially mostly concerned with cultivation of *Escherichia coli*[7, 13–17], high cell densities have been achieved with several other organisms as well with improved product accumulation. The bacterium *Bacillus megaterium*[18], the yeasts *Rhodococcus opacus*[19], *Pichia pastoris*[20] and *Candida rugosa*[21], the diatom *Nitzchia laevis*[22], the algae *Chlorella vulgaris*[23] and *Nannochloris atomus* Butcher[24], the plant cells *Panax notoginseng*[25] and other cell cultures[8] have shown enhanced cell densities. The specific details of several HDC studies and their product yields are in Table 1.

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Table 1 – *High-density cultivations adopted for different product formations: The maximum cell mass and product obtained from different species*

Cell	Product	Cell mass	Product	Method	Ref.
Methylobacterium extorquens	Poly(3-Hydroxybutyrate)	115 g L^{-1}	52.9 g L^{-1}	OTR based fed-batch	73
Zobellella denitrificans MW1	Poly(3-Hydroxybutyrate)	54 g L^{-1}	36.4 g L^{-1}	Medium-optimized fed-batch cultivation	96
Bacillus megaterium BA-019	Poly(3-hydroxybutyrate)	72.6 g L^{-1}	30.5 g L^{-1}	Medium-optimized fed-batch cultivation	18
Ralstonia eutropha	Poly(3-Hydroxybutyrate)	300 g L^{-1}	N/A	DO stat fed-batch	12
Rhodococcus opacus PD630	TAG	77.6 g L^{-1}	27.6 g L^{-1}	Medium-optimized batch culture	19
Crypthecodinium cohnii	Lipid Docosahexanoic acid	109 g L^{-1}	61 g L^{-1} 19 g L^{-1}	DO stat fed-batch	77
Nitzschia laevis	Eicosapentanoic acid	40 g L^{-1}	1.1 g L^{-1}	Perfusion fed-batch	22
Saccharomyces cerevisiae	Ergosterol	120 g L^{-1}	1500 mg L^{-1}	Glucose-controlled fed-batch cultivation	97
Kluyveromyces marxianus	Ethanol	60 g L^{-1}	16 g L^{-1}	pH stat fed-batch	85
Saccharomyces cerevisiae KV-25	Biomass	187.63 g L^{-1}	N/A	Medium-optimized fed-batch cultivation	93
Candida utilis CBS 621	Biomass	90 g L^{-1}	N/A	Maltose feedstock	51
Chlorella sp. NCTU-2	Biomass	5.15 g L^{-1}	N/A	Porous centric-tube photobioreactor	90
Taxus chinensis	Paclitaxel	22 g L^{-1}	137.5 mg L^{-1}	Intermittent feeding fed-batch	98
Pichia pastoris	Human cystatin	125 g L^{-1}	0.78 g L^{-1}	Fed-batch cultivation	20
Pichia pastoris	Hepatitis B surface antigen	97 g L^{-1}	0.2 g L^{-1}	Continuous fermentation	99
Methylobacterium extorquens	Green Fluorescent Protein	56 g L^{-1}	4 g L^{-1}	OTR based fed-batch	10
Escherichia coli	Green Fluorescent Protein	90 g L^{-1}	1.3 g L^{-1}	Fed-batch cultivation	38
Escherichia coli	Recombinant protein	172 g L^{-1}	0.3 g L^{-1}	Fed-batch cultivation	100
Escherichia coli	Recombinant protein	70 g L^{-1}	6 g L^{-1}	Fed-batch cultivation with stress minimizing method	64
Escherichia coli	Recombinant protein	15 g L^{-1}	N/A	Enzymatically controlled fed-batch cultivation	34
Escherichia coli	Recombinant protein	20 g L^{-1}	N/A	Rocking motion type bioreactor	17
Pichia pastoris	Recombinant protein	>200 g L^{-1}	6.5 g L^{-1}	Glucose-limited fed-batch cultivations	11
Kluyveromyces marxianus	Betagalactosidase	105 g L^{-1}	2.2 g L^{-1}	Fed-batch	9
Hansenula polymorpha	Levansurcrase	120 g L^{-1}	12.2 U mL^{-1}	DO stat fed-batch	87
Brevibacterium linens	Proteinase	50 g L^{-1}	0.2 U mL^{-1}	Transitional feeding	86
Kluyveromyces marxianus	β-Galactosidase	105 g L^{-1}	5.7 U mg^{-1}	Exponential feeding fed-batch	101
Ralstonia eutropha	Organophosphohydrolase	182 g L^{-1}	4.3 g L^{-1}	Fed-batch cultivation	102
Pichia pastoris	Alpha amylase	110 g L^{-1}	340 mg L^{-1}	DO stat fed-batch	103
Pichia pastoris	Bovine entrokinase	45 g L^{-1}	9000 U mg^{-1}	pH stat fed-batch	104
Pichia pastoris	Lipase	50.4 g L^{-1}	385 AU mL^{-1}	Specific growth rate control	105
Candida rugosa	Lipase	5.9 g L^{-1}	117 U mL^{-1}	Constant feeding	21
Yarrowia lipolytica	Lipase	1.79 g L^{-1}	2016 U mL^{-1}	Fed-batch cultivation	57
Candida cylindracea	Lipase	90 g L^{-1}	23.7 U mL^{-1}	Specific growth rate control	56
Schizosaccharomyces pombe	Lipase	>50 g L^{-1}	16 U mL^{-1}	Specific growth rate control	83
Hansenula polymorpha	Human serum albumin	83 g L^{-1}	550 mg L^{-1}	DO stat fed-batch	106
Escherichia coli	Gluthathione	80 g L^{-1}	880 mg L^{-1}	Exponential feeding fed-batch	107
Panax notoginseng	Ginseng saponin	27.3 g L^{-1}	0.55 g L^{-1}	Fed-batch	25

For a HDC to be viable, the species must be capable of growing within process equipment constraints (such as heat and oxygen transfer capacities, mixing, shear, etc.), as well as tolerating any metabolic products formed. The advantages of HDC include increased cost effectiveness, reduced culture volume, enhanced downstream processing, reduced wastewater production, lower production costs, and reduced capital requirements[13]. The problems associated with HDC include product inhibition[26–28], limited oxygen[29] and heat transfer[30] capacities of processing equipment, and formation of unwanted by-products[26,31–33]. While the problems may seem substantial, many of them can be overcome through careful considerations in the design and operation of the process. This review focuses on recent approaches utilized in HDC as well as to solve the problems that have been associated with HDC to achieve increased productivity either through increased cell density or specific productivity or both. The primary focuses of this review are on the medium composition, modes of operation and feeding schedules, reactor conditions such as oxygen transfer, temperature, agitation, and pH. Furthermore, actual research about the stress reaction of the utilized microorganisms are discussed to improve HDC processes.

Cell growth and medium optimization

Microorganisms experience various limiting conditions when grown as HDC. Cells in high-density cultures face conditions such as nutrient depletion, elevated osmotic pressures, increased viscosity, limited oxygen transfer capacity, and local as well as temporal variations in concentrations of nutrients, temperature, agitation, and pH. Proportionately large amounts of the substrate and nutrients are required for achieving high cell densities and increased product concentrations. Even if the requisite amounts can be incorporated in the medium at one time, the high concentrations may be toxic to cell growth. For example, *E. coli* growth was inhibited[27] when the concentration of glucose, ammonia, iron, magnesium, phosphorus, and zinc exceeded, respectively, 50 g L^{-1}, 3 g L^{-1}, 1.15 g L^{-1}, 8.7 g L^{-1}, 10 g L^{-1} and 0.038 g L^{-1}. Therefore, controlled feeding strategy is usually employed as described further herein. Additionally, excess carbon source can result in formation of metabolic by-products that can inhibit cell growth and/or reduce carbon efficiency. Some common metabolic byproducts are acetate for *E. coli*, propionate for *Bacillus* spp., ethanol for *S. cerevisiae*[33], and exopolysaccharides for *Lypomyces starkeyi*[31,32]. In high-density cultivation of *Lypomyces starkeyi*, exopolysaccharide production was observed when the glucose concentration exceeded 90 g L^{-1} [31,32]. Maintaining a low concentration of glucose through intermittent or continuous feeding can mitigate this byproduct formation or minimize it.

Of course, strains capable of withstanding high substrate, nutrient, and/or product concentrations are very desirable or even necessary as in the case of *S. cerevisiae,* for which the product itself becomes inhibitory when its exogenous concentration exceeds 70 g L^{-1} [26]. Kurosawa *et al.*[19] reported that *Rhodococcus opacus* PD630 could grow in defined media supplemented with glucose up to a concentration of 300 g L^{-1} in batch culture. *R. opacus* PD630 grown in batch-culture with an optimal production medium containing 240 g L^{-1} glucose and 13.45 g L^{-1} $(NH_4)_2SO_4$ (for C/N ratio of 17.8) yielded 77.6 g L^{-1} of cell dry weight composed of approximately 38 % TAGs.

For small-scale batch operation, several technologies involving novel growth media have been introduced[16,17,34]. Herein, slow release of growth substrates is achieved through use of slow-release polymers (FeedBeads®, FeedPlates®) or through enzymatic degradation of polymers (EnBase® technology). Using a complex medium supplemented with peptone and yeast extract, 10-fold increase in volumetric productivity over conventional Luria broth medium for production of recombinant proteins by *E. coli*1 has been reported using the EnPresso® system[16,34]. An extensive review of these 'intelligent media' for production of recombinant proteins has been provided by Krause *et al.*[34]

However, the use of complex medium components such as peptone and yeast extract is often discouraged since their composition and quantity can vary from batch to batch, and thus pose issues of reproducibility in production processes. Media with known nutrient composition permit accurate control of cellular environment[35] and are, therefore, favored in industrial large-scale operations. This is often combined with supplementation of certain amino acids to improve productivity. In the case of *E. coli*, β-isopropylmalate dehydrogenase activity was improved with the addition of a dose of 1 g L^{-1} leucine at the beginning of the culture[36]. *S. cerevisiae* has also shown improved protein production in a defined medium supplemented with alanine, arginine, asparagine, glutamic acid, glutamine, and glycine[37]. However, the value of the product and cost factor must be considered when adding amino acids.

Use of defined media is, however, not without its own pitfalls, and requires medium optimization and tweaking of feeding strategies in high-density cultivations. As an example, Beckmann *et al.*[38] reported Leucine was inhibitory to leucine-auxotrophic *Escherichia coli* K12 ER2507 even at a concentration of 0.3 g L^{-1}. This effect could be overcome by optimizing the feeding strategy and medium

composition. Lau *et al.*[39] reported that high cell-density fed-batch bioprocess for the heterologous production of 6-deoxyerythronolide B in *Escherichia coli* using a defined medium resulted in accumulation of excess. Following optimization of the medium composition and fermentation conditions, the maximum cell density was increased by two-fold. Overall, the fed-batch process represented an 11-fold improvement over the batch process. This was achieved by maintaining the glucose and propionate levels in the bioreactor between 5 and 15 g L^{-1}, and 20 and 40 nM, respectively, and by feeding 565 mmol d^{-1} ammonium sulfate.

Selection and optimization of appropriate medium components is critical to successful operation of HDC. During high-density cultivation, the cells may experience environmental stress, limitations of nutrients, and even changes in metabolic states. Hence, the medium should be optimized to ensure that all the nutrients are available in sufficient quantities for cellular growth and suitable for downstream processing. Burgard *et al.*[40] found that methanol-and glucose-grown cultures of *Pichia pastoris* experienced transcriptional upregulation of genes connected to phosphorus and sulfur metabolism from the middle to the end of the fed-batch. The genes connected to transport processes utilized alternative substrates to cover the phosphorus nitrogen, and sulfur limitation. Addition of proper supplementation countered the upregulation resulting in significant improvements in cell growth and recombinant protein expression. Selection of appropriate substrate too is critical. As an example, although methanol or methanol/glycerol mixtures have been used for production of heterologous proteins in *Pichia pastoris* in high-density cultivations, using methanol as carbon source has potential for cell lysis and explosion hazard during commercial operation[11]. To avoid this, methanol may be substituted with glucose as sole carbon source in high-density cultivation of *P. pastoris* for recombinant protein production as demonstrated by Heyland *et al.*[11] Using a recombinant *P. pastoris* strain in glucose-limited fed-batch cultivations, over 200 g L^{-1} of dry cell weight (CDW) concentration was achieved resulting in a recombinant protein, Bap A, titer of 6.5 g L^{-1}.

Another consideration in the preparation of media is the solubility of certain medium ingredients, especially when they are added in large concentrations. Presence of phosphates, sulfates, chloride, sodium, calcium, ammonium, and magnesium in the medium has the possibility of forming non-soluble complexes such as ammonium phosphate, magnesium phosphate, calcium phosphate, and other metal phosphates during sterilization as well as during the fermentation process due to the production of organic acids and carbon dioxide. These complexes may precipitate[14]. Precipitation can pose an issue when it comes to downstream processing, such as purification as well as monitoring devices. Precipitation can also complicate the process of measurement and monitoring not only mineral concentrations but also cell densities[41]. Furthermore, high salt concentrations pose additional issues, such as osmotic pressure and conductivity that affect membrane stability and may result in a reduction of growth rate[42].

Brady *et al.*[43] presented a simplistic solution to such issues by simply reducing the salt concentrations to a quarter of the recipe of the medium, although that is not equally applicable to many species. As salt precipitation is a common problem during *P. pastoris* high-density fermentation due to the accumulation of certain lipids, Brady *et al.*[43] used a low-salt, fully defined medium for the production of the full-length P30P2MSP1-19 (a recombinant subunit vaccine for malaria) in *Pichia pastoris*, that did not reduce growth rates or biomass yields on either glycerol or methanol utilized to avoid precipitation. A total of 500 mg L^{-1} of secreted purified protein was produced in high cell density fermentation from a wet cell weight of 180 g L^{-1}. Similarly, Yup *et al.*[44] reported that, decreasing the phosphorus concentration by reducing initial KH_2PO_4 (from 7.5 g L^{-1} to 4 g L^{-1}) during a fed-batch culture of *Pseudomonas putida,* could result in increased formation of polyhydroxyalkanoate (from 32.3 g L^{-1} to 72.6 g L^{-1}). Conversely, Lau *et al.*[39] doubled the concentration of phosphate, in addition to a propionate-controlled feed, to achieve doubled cell concentrations and an eleven-fold increase in production of 6-deoxyerythronolide B.

Identification and delivery of appropriate nutrients is critical to achieving high product titers in high-density cultivation. As an example, López *et al.*[45] found that, although a relatively high cell density (29.9 g L^{-1}) of *Zobellella denitrificans* MW1 could be obtained in only a short period (24 h) during fed-batch operation feeding glycerol, the production of Poly(3-Hydroxybutyrate) was relatively low (9.3 g L^{-1}). Much higher concentrations of PHB (up to 54.3 g L^{-1}) and higher cell densities (up to 81.2 g L^{-1}) were obtained by fed-batch operation in the presence of 20 g L^{-1} NaCl coupled with optimized feeding of glycerol and ammonia to support both cell growth and polymer accumulation over a period of 50 h. A high specific growth rate (0.422 h^{-1}) and a short doubling time (1.64 h) were attained. Similarly, Kim *et al.*[46] utilized an ammonia-restriction feeding strategy to enhance the PHB production in a fed-batch culture of *Alcaligenes eutrophus* with broth glucose concentration control in the range of 10 to 20 g L^{-1}. The final cell concentra-

tion, PHB concentration and PHB productivity increased as feeding of ammonia was stopped at the cell concentration of 70 g L^{-1} (cell yield ~0.43 g g^{-1} glucose). Final concentrations of PHB (121 g L^{-1}) and total cells (164 g L^{-1}) were obtained in 50 h (cell yield ~0.38 g g^{-1} glucose). For the production of a polyhydroxyalkanoate (PHA) using *Ralstonia eutropha*, Riedel *et al.*[47] developed a high cell density cultivation fed-batch process using fructose or palm oil as a carbon source and nitrogen limitation to induce PHA accumulation. The feed of carbon source was ~170 g L^{-1}. This fermentation resulted in a biomass concentration of 139 g L^{-1} cell dry weight with 74% of dry cell weight as PHA containing 19 mol% poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) with PHA productivity of 1 g L^{-1} h^{-1}.

Thus, media optimization and the optimization of the feeding profile can be a highly valuable avenue of research in establishing the optimal conditions to obtain HDC. While early approaches were trial and error processes, statistical techniques such as Response Surface Methodology (RSM) or factorial design can provide a more organized avenue for the Design of Experiments (DOE). Beckmann *et al.*[38] applied full factorial design of experiments to optimize the concentration of glucose and leucine in the feed medium and achieved a cell density of 97 g L^{-1} for the leucine auxotrophic *E. coli* strain K12 ER2507. Subramaniam *et al.*[48–50] developed a minimal medium by replacing costly yeast extract with vitamins such as biotin, calcium pantothenol and inositol. They utilized RSM to optimize the concentrations of vitamins and phosphates. With the optimized minimal medium, Subramaniam *et al.*[48–50] could reduce the medium cost for lipid production by *Lipomyces starkeyi* from $12.68 per gallon lipids to $3.11 per gallon lipids with high cell density of 44.5 g L^{-1} and lipid concentration of 33.5 g L^{-1}.

Reactor operation

Since the environmental conditions importantly affect cell growth as well as product formation, the success of high-density cultivation is dependent on choosing an optimal method of reactor operation. Several different methods have been developed for supplying nutrients to cells in culture. The key consideration when choosing a method is to ensure that the concentrations of substrate(s) and nutrients are maintained within the desired range to avoid overfeeding or underfeeding. While batch[51] and continuous mode of operation have been utilized, fed-batch operation is most commonly used in HDC.

Fed-batch operation

Fed-batch cultivation involves a semi-batch operation of bioreactor in which the nutrients necessary for cell growth and product formation are fed continuously or intermittently into the reactor. The culture broth is typically harvested only at the end of the operational period, either fully or partially, with the remainder serving as the inoculum for the next run. Through the manipulation of feeding and withdrawals, the fed-batch operation can provide unique means of regulating the broth concentration of compounds that control the key metabolic pathways and, therefore, possess definite advantages over batch or continuous operation. The fed-batch process is particularly useful for HDC due to the control of substrate concentration that is necessary, as well as increasing the working time for growth-related product formation.

Shiloach and Fass[14] have discussed several different strategies that can be used to achieve HDC for *E. coli*. Continuous and constant rate of feeding utilizes addition of nutrients into the bioreactor at a specified rate. Under substrate-limited conditions of growth in such feeding strategy, the specific growth rate of cells decreases, and cell concentration increases also slow down[52] since the volume of reactor contents increases constantly. On the other hand, variable feed rates may provide some advantages depending on the situation. Jensen and Carlsen[52] reported that a gradual increase in the feeding rate could mitigate the effects of increased volume and enhance cell growth by supplying more nutrients. A feeding rate (of the limiting substrate) that is related to the cell concentration can allow for an extension of the exponential growth phase. This exponential feeding method can be used to allow cells to grow at constant specific growth rates.[14] When the growth characteristics of an organism are known, the exponential feeding rate can be tailored to provide the optimal metabolic condition, resulting in maximal productivity[53–55]. Kim and Hou[56] compared intermittent feeding of oleic acid (where the substrate was added to bring its concentration back to initial concentration after a certain time) with stepwise feeding (designed to simulate exponential feeding to support specific growth rate of cells) in fed-batch cultures of *Candida cylindracea* to improve cell concentration and lipase activity. In intermittent feeding with oleic acid added after 26.5 h from cultivation start to the initial oleic acid concentration of 50 g L^{-1}, the final cell concentration was 52 g L^{-1} and the extracellular lipase activity was 6.3 U mL^{-1} at 138.5 h. In stepwise feeding simulating a constant specific growth rate of 0.02 h^{-1}, the highest final cell concentration obtained was 90 g L^{-1} and the extracellular lipase activity of 23.7 U mL^{-1}, showing clearly the importance of selecting the optimal feeding strategy for maximal product formation.

Feeding an inducer at appropriate stages of fed-batch operation is commonly employed to enhance expression of products. Fickers *et al.*[57] reported that feeding of complete glucose medium in a fed-batch culture of *Yarrowia lipolytica* resulted in a 2-fold increase in lipase production compared to batch cultivation. But when olive oil was also added to the glucose medium during feeding, the lipase production increased three-fold that of batch culture. In another study, intermittent feeding of 12 % glycerol solution resulted in 83 g L^{-1} dry cell weight with human serum albumin production of 550 mg L^{-1} in less than 32 h of fed-batch cultivation of *Hansenula polymorpha* having the glyceraldehyde-3-phosphate dehydrogenase promoter to express HSA[58]. Joo-Hyung *et al.*[58] also reported that addition of glycerol was more stimulatory to the production than having only glucose or methanol. Similarly, Choi *et al.*[59] also noted that paclitaxel production in suspension culture of *Taxus chinensis* cells could be enhanced by utilizing an intermittent feeding strategy of sucrose, but intermittent feeding of maltose as carbon source was much more stimulatory. Twenty-six milligrams per liter paclitaxel production was achieved through the intermittent feeding of 3, 1, and 2 % (w/v) sucrose at 0, 7, and 21 days, respectively. Sixty-seven milligrams per liter paclitaxel production was achieved through the intermittent feeding of 1 and 2 % (w/v) maltose at 7 and 21 days, respectively. On the other hand, use of fructose in high-density cultures did not prove beneficial.

Two-stage cyclic fed-batch operation

Shin *et al.*[60] studied the production of human mini-proinsulin through a two-stage fed-batch fermentation process to achieve a high cell density of *Escherichia coli.* This two-stage fed-batch operation, a modification of the fed-batch process, consisted of a growth tank and an induction tank where the product formation took place. The cells were grown in the growth tank, and a portion of the cell broth was transferred to the induction tank, while leaving a small volume of cell broth in the growth tank for continued cell growth. The volume of liquid in the growth tank was refilled to its pretransfer volume while product formation was taking place in the induction tank. By separating the growth and production phases of cell cultivation, the culture conditions, such as temperature, pH, and medium compositions could be defined to a state ideal for each process. By applying this two-stage fed-batch fermentation process to produce human mini pro insulin by *Escherichia coli*, up to 44 % increase in volumetric productivity was achieved from single-stage fed-batch fermentation where productivity was 0.175 g L^{-1} h^{-1}. Such a process design is very attractive as it may lead to a continuous fermentation process for recombinant DNA products with decreased down-times and reduced downstream costs. FDA encourages pharma companies to develop continuous processes to achieve higher product quality[61]. In the case of *Yarrowia lipolytica* SMY2, the use of this process led to a two-fold increase in volumetric productivity of rice α-amylase production compared to a conventional fed-batch reactor[21]. However, these benefits need to be balanced with increased complexity of operation, potential for contamination, losses due to strain instability, and ultimately increases capital costs.

Temperature-limited fed-batch reactor

A unique approach to the fed-batch process is the use of temperature to control growth as opposed to the conventional use of substrate feeding rates. By utilizing a gradual decrease in temperature, Jahic *et al.*[62] found that a higher accumulation of protein can be achieved through to a combination of reduced protein inactivation due to thermolysis and reduced cell death resulting in reduced proteinase release. De Maré *et al.*[29] used cultivation combining a temperature-limited fed-batch process with feed control to achieve a 20 % increase in cell mass. Temperature gradients may also lead to a higher fraction of soluble proteins since high temperatures cause a high amount of inclusion bodies and low temperatures result in a higher amount of soluble fraction[63].

Temperature control is already of major concern in industrial applications of microbial fermentation, and it poses a much more important role in high-density cultivation due to two factors that are directly caused by high-density cultivation: increased heat generation, and reduced heat transfer. The increase in heat generation is simply a function of the increased cell density, resulting from the metabolic processes, while the reduction in heat transfer is caused by an increase in viscosity that tends to accompany high-density cultivation. Donovan *et al.*[30] demonstrated that temperature affects the yield of proteins in culture, and a reduction in culture temperature can be a simple method of increasing protein production. Also, Wyre and Overton[64] suggest an adapted temperature profile for the improved production of soluble proteins by the reduction of stress through reduced temperature during the production of recombinant proteins.

Dialysis reactors

In the case of unavoidable inhibitory effects of nutrients or byproducts, a method to reduce the effective concentrations of the substances can prove beneficial. Dialysis can be a way to overcome this limitation. Dialysis is the separation of broth com-

ponents dissolved in a liquid by differences in their ability to pass through a semi-permeable membrane driven by a concentration differential. Shiloach and Fass[14] have reported two such reactor configurations to achieve high-density cultivations. One design utilizes a separate reactor and reservoir connected by a dialysis machine, and the other has a single reactor that uses a membrane to create two compartments. Nakano *et al.*[65] reported that a membrane dialysis bioreactor was able to achieve *E. coli* cell density of 190 g L^{-1}. Fuchs *et al.*[66] argued that a single vessel is not very viable due to sterilization difficulties, as well as sensitivity to mechanical stress during stirring and oxygen transfer limitations. Using an external membrane module, Fuchs *et al.*[66] demonstrated that the lab-scale dialysis reactor cultivation of *E. coli* could be replicated on an industrial scale. A 300-L reactor using this external membrane dialysis technique was able to achieve cell densities like those in conventional fed-batch (190 g L^{-1}), resulting in a 3.8-fold increase in the production of protein.

Pressurized reactor

Since oxygen transfer can be critical in HDC, methods to improve the efficiency of oxygen transfer can be lucrative for the cultivation process. High-density cultivation of *E. coli* revealed that pressurized reactors can be a method to meet the high demand of oxygen[67]. Gas flow rate management along with increases in pressure allowed for mitigation of the carbon dioxide inhibition that developed, allowing for a final cell density of 130 g L^{-1}, with the increased tryptophan synthase specific activity of 160 U mg^{-1}-protein which was 40-fold higher than obtained with non-recombinant *E. coli* cells.

Control and operating parameters

There are four basic approaches to implementing the feeding schedules:

1. The open-loop control scheme.
2. Indirect control of substrate based on the non-feed parameter.
3. Indirect control based on mass balance equations.
4. Direct control based on on-line substrate measurements.

Measurements can be done *off-line*, *semi-online* or *online*. On-line measurements offer better and more flexible control of the feeding. The operation of the reactor is strongly influenced by the measurement possibilities and the mathematic model of the process[68,69]. The type and quality of sensors available is a major consideration for the determination of the control scheme.

Open-loop process control can be used when robust mathematical models for the process are available. However, it suffers from not being able to respond to any unexpected conditions during a fermentation process. An indirect feedback control utilizes an observable parameter that is closely related to the course of microbial fermentation, such as dissolved oxygen, pH, carbon dioxide partial pressure, or culture fluorescence. These readings can be used as an indication of the state of the fermentation process. The direct feedback control is the most accurate of the control schemes. It uses the concentration of the limiting substrate in the culture medium as the parameter. However, there is much difficulty in its use, since obtaining accurate on-line substrate measurements can be complicated by the scarcity of sensors that can measure many compounds. However, in the cases where it is utilized, it provides a controlled process, that can respond very well to unexpected conditions[70,71].

The green fluorescent protein (GFP) was used as a model protein to study the recombinant protein production by the strain *Methylobacterium extorquens* ATCC 55366 by Béland *et al.*[72] Scale-up from shake flasks to 20-L fed-batch fermentation was achieved with methanol as a sole carbon and energy source in a minimal medium. The methanol concentration was monitored with an on-line semiconductor gas sensor in the culture broth. It was maintained at a non-toxic level of 1.4 g L^{-1} with an adaptive control which regulated the methanol feed rate. A maximum growth rate of 0.18 h^{-1} with an overall cell yield of 0.3 g g^{-1} methanol was achieved, producing GFP that accounted for 16 % of cell protein content.

Similarly, Bourque *et al.*[73] revealed that *Methylobacterium extorquens* ATCC 55366 can be successfully cultivated at very high cell densities in a fed-batch fermentation system using methanol as a sole carbon and energy source in a minimal medium to produce poly b-hydroxybutyrate (PHB). Cell biomass levels were between 100 g L^{-1} and 115 g L^{-1} (dry weight) and cells contained between 40 % and 46 % PHB on a dry-weight basis. Controlling the methanol concentration at a very low level (less than 0.01 g L^{-1}) during the PHB production phase, led not only to prevention of oxygen limitation but also to the production of very high-molecular-mass PHB, in the 900–1800 kDa range.

When investigating the enhancement of *Candida rugosa* lipase production using different control fed-batch operational strategies, Gordillo *et al.*[21] compared constant substrate feeding rate strategy with feeding-rate control based on a constant specific growth rate and found that maximum lipase

production by *Candida rugosa* with a constant specific growth rate strategy was twice that using a constant substrate feeding rate. The isozymes produced using constant specific growth rate strategy were three to four times more active compared to those with constant feeding rate strategy. On-line carbon dioxide evolution rates (CER) were used to evaluate cell specific growth rates using a mathematical model based on continuous culture studies.

Feedback control can also be implemented to dual-level systems to obtain finer control action. In the case of a fed-batch culture of recombinant *E. coli*, Turner *et al.*[74] found that higher cell concentrations with limited by-product formation can be achieved by using a two-level control scheme that measured galactose (carbon substrate) and acetate (by-product) concentrations and adjusting feed rates accordingly.

The parameters such as pH, temperature, and dissolved oxygen affect the cell growth and product formation, which needs to be controlled at optimum values. The control parameters may also be switched based on the reactor conditions and specific to process as different parameters need to be controlled at different stages of bioprocesses. In the case of high-density cultivation of baker's yeast, the substrate concentration needs to be controlled during the initial stages of fermentation to avoid formation of by-products, but oxygen transfer becomes limiting at the later stage of fermentation upon reaching the high cell density, and dissolved oxygen is the one that needs to be controlled[75]. The choice of parameters is dependent on the system, and decisions are frequently made based on convenience and prior experimental observations. Mathematical models can be used to estimate the quality of the controlling variables and combined with the availability of sensors; the reactor operation can be dictated.

Calorimetry

Calorimetry is a basic control tool that can be used in an indirect control scheme. It involves measurement of heat produced during the microbial growth process. It is an easily performed continuous *on-line* measurement that can be done without disturbing the culture. Its main advantage lies in the generality since all microbial processes generally produce heat. It can be replaced by exhaust gas analysis unless the process is anaerobic without gaseous product formation. Larsson *et al.*[76] described how to use the heat production rate, measured by a flow-through microcalorimeter as the only continuous *on-line* measurement, in order to indirectly measure the substrate and product concentrations during aerobic fed-batch growth of *S. cerevisiae*; use of this measurement reduced the maximum ethanol concentration from 2 g L^{-1} during batch growth to 0.2 g L^{-1} using the same amount of glucose (5 g L^{-1}).

pH

pH is a commonly used control parameter that can be used to measure the acid production by growing cells. De Swaaf *et al.*[77] used both a constant glucose concentration feeding strategy with *Crypthecodinium cohnii* to produce docosahexaenoic acid, as well as an acetic acid feeding strategy that was controlled by culture pH. A feed consisting of acetic acid (50 % w/w) resulted in a higher overall volumetric productivity of DHA (rDHA) than a feed consisting of 50 % (w/v) glucose (38 and 14 mg L^{-1} h^{-1}, respectively). The rDHA productivity was further increased to 48 mg L^{-1} h^{-1} using a feed consisting of pure acetic acid. The latter fermentation strategy resulted in final concentrations of 109 g L^{-1} dry biomass, 61 g L^{-1} lipid, and 19 g L^{-1} DHA, which were some of the highest values reported for a heterotrophic alga.

Files *et al.*[20] showed the contrasting effects that pH can have on the cultivation process. A *Pichia pastoris* fermentation process for producing high levels of recombinant human cystatin-C was investigated at multiple pH conditions, and it was found that, although cell density increased as pH increased, active cystatin-C yields dropped. Thus, it is important to consider the conflicting results of the variable to optimize the culture. The results of the fermentation of *Pichia pastoris* are of great importance because *Pichia* strains are used very often for recombinant protein production in biotechnological processes[78].

Chen *et al.*[79] showed how rhamnolipid production by *Pseudomonas aeruginosa* S2 can be enhanced, in addition to increased cell concentrations, by the pH stat control of feeding, as opposed to constant feeding. Batch fermentation using optimal medium and operating conditions resulted in rhamnolipid production of 5.31 g L^{-1} by *P. aeruginosa* S2 in a glucose-based medium. Constant feeding of glucose increased that maximum rhamnolipid concentration to 6.06 g L^{-1} with 6 % glucose feed. Combining a repeated fed-batch culture with the pH-stat led to a marked increase in the rhamnolipid production, resulting in a yield of 9.4 g L^{-1}.

During the fermentation of lactic acid bacteria, large amounts of acids are produced which result in an inhibition of microbial growth after reaching a certain concentration. With the use of adaptive laboratory evolution, genome shuffling, and synthetic biology, new strains have been created with improved acid resistance to withstand low pH values and thus ability to achieve higher cell concentra-

tions[80]. Increased acid tolerance of *Lactobacillus casei* resulted in 60 %, 13.6 %, and 65.6 % increases in concentrations of biomass, lactate and acetate, respectively, when cultured at pH 4.3 for 64 h[81].

Respiratory quotient

Respiratory Quotient (RQ) is the ratio of carbon dioxide production rate and oxygen consumption rates in a bioreactor, which can be measured online rapidly using gas analyses. This dimensionless number is a measure of basal metabolic rate in the broth and it is a form of indirect calorimetry. Jong *et al.*[82] used RQ as control parameter to manage glucose concentrations and enhance production of thuringiensin by *Bacillus thuringiensis* in a modified airlift reactor. While batch culture with similar glucose conditions achieved a maximum of 1.7 g L^{-1} thuringiensin production with $6{\cdot}10^{12}$ cells per L, the fed-batch operation with glucose concentration controlled by the RQ method at 3 g L^{-1} achieved thuringiensin concentrations of 11.71 g L^{-1} and $4{\cdot}10^{13}$ cells per L with a yield of 0.0905 g thuringiensin g^{-1} glucose. This is a significant improvement over the batch culture.

Ikeda *et al.*[83] also used a RQ-dependent feedback regulation to avoid glucose effect from overfeeding in the production of Recombinant Human Lysosomal Acid Lipase in *Schizosaccharomyces pombe* in high-density cultivation. The nutrient supply was automatically initiated and controlled based on changes in respiratory quotient as glucose in broth was consumed in batch-phase culture achieving >50 g L^{-1} of dry cell weight L^{-1} and human lysosomal lipase activity of 16000 U L^{-1}.

Dissolved oxygen

Many bioprocesses are aerobic requiring above-critical levels of dissolved oxygen in the broth. This is the major limiting factor in HDC due to the low solubility of oxygen in the culture liquids. At standard temperature and pressure (STP 25 °C, and 1 atm), the saturation dissolved oxygen concentration in medium sparged with atmospheric air is around 8 mg L^{-1}. Although the oxygen supply can be increased by changing the aeration rate and agitation speed[55], given the reactor design including impeller type and dimensions, oxygen can still become limiting in HDC. Dissolved oxygen concentrations below critical levels result in reduced specific growth rates, shifting metabolism, and formation of several unwanted metabolites arising from mixed acid metabolism, such as acetate, lactate, and ethanol[84,82]. This issue may be mitigated by use of enriched (even pure) oxygen and/or pressurizing the reactors. Since the dissolved oxygen concentration at saturation is proportional to the partial pressure of oxygen (Henry's Law), both the methods result in increased oxygen supply to the cells.

Belem and Lee[85] investigated oligonucleotide production in fed-batch fermentation of cheese whey with *Kluyveromyces marxianus* during high-density cultivation. While yields and specific growth rates in batch operation were higher than in fed-batch operation, productivities were higher during in fed-batch reactor, especially when the aeration rate was increased to 2 vvm.

Adamitsch *et al.*[86] used oxygen-enriched air supply to achieve increased oxygen transfer. By the addition of pure oxygen supplement to the air inlet, a minimum of 20 % dissolved O_2 saturation was maintained. This allowed the cultivation of *Brevibacterium linens* to cell density reaching 50 g L^{-1} in 60 h, with the volumetric productivity of the proteolytic and lipolytic activities 4220 U L^{-1} h^{-1} and 7.3 U L^{-1} h^{-1}, respectively. The overall enzyme yield coefficient on biomass had not attained the level of $18.7{\cdot}10^3$ U g^{-1}, which was determined in a batch cultivation yielding 1.9 g L^{-1} bacterial biomass, but the volumetric productivity in high-cell-density culture ($4.2{\cdot}10^3$ U L^{-1} h^{-1} at 55 h) exceeded considerably that of the volumetric productivity of a batch fermentation ($1.8{\cdot}10^3$ U L^{-1} h^{-1}).

Park *et al.*[87] utilized a strategy of supplying high-purity oxygen to increase the viable cell density in a bioreactor and enhance the production of the target protein in the production of human serum albumin by the fed-batch culture of *Hansenula polymorpha*. To supply high-purity oxygen to the bioreactor, nearly 100 % high-purity oxygen from a commercial bomb or higher than 93 % oxygen available in situ from a pressure swing adsorption (PSA) oxygen generator was employed. With high-purity oxygen, the final *H. polymorpha* cell densities and the HSA concentrations were 24.6 g L^{-1} and 5.1 g L^{-1} in the 5-L fermenter, and 24.8 g L^{-1} and 4.5 g L^{-1} in the 30-L fermenter, respectively. These were 2–10 times higher than those obtained in air-sparged fed-batch fermentations.

Dissolved carbon dioxide

Carbon dioxide is the final product of the breakdown of carbon sources utilized for energy purposes. HDC increases carbon dioxide production rates, and thus potential for high dissolved carbon dioxide concentrations. While the carbon dioxide is by itself inhibitory to cell growth[55], it also results in the production of acetate and carbonate, which can result in acidification of the medium. Castan *et al*.[84] reported the toxic effects of carbon dioxide resulting in growth inhibition. As a gas, carbon dioxide also follows Henry's Law regarding partial pressures and dissolved concentration. This results in a

dilemma on the use of the pressurized reactor, which in addition to increasing the oxygen supply, may also enhance the effects of carbon dioxide[67]. But there are also positive effects of carbon dioxide on microbial growth and product formation[88,89]. These examples of the positive and negative effects of CO_2 indicate the importance of the consideration of CO_2 concentrations in bioreactors.

Chiu *et al.*[90] investigated carbon dioxide sequestration in photobioreactors growing high-density cultures of microalgae. Three types of photobioreactors were tested: (i) without inner column (i.e., a bubble column), (ii) with a centric-tube column, and (iii) with a porous centric-tube column. The porous centric tube column had the highest specific growth rate for *Chlorella* sp. NCTU2. Their results showed that the CO_2 removal efficiency was related to biomass concentration and aeration rate.

Agitation

A consequence of the high viscosity exhibited by HDC is difficulty in mixing the content of the bioreactor, which increases with increasing reactor size[27]. As a result, large-scale reactors have gradients of concentrations due to incomplete mixing. This can result in cells experiencing toxic concentrations of substrate in certain zones, while others may be nutrient-starved. Furthermore, even when the cells do circulate through these zones, they may experience stress as a result of the varying environmental conditions. Kapat *et al.*[91] suggested that increasing the rate of agitation can lessen these issues, enhancing oxygen transfer coefficient and increasing protein production. However, the application of this technique may be limited to those cell types that can withstand the shear stress generated due to the increased agitation, while other cell types may undergo lysis as a result[92]. Also, foam formation is intensified with increased agitation speeds. Increasing cell and product concentrations also enhance foam production. Since excessive foaming can interfere with normal operation of aerated stirred reactors, foam reduction measures must be considered when designing the bioreactor for HDC.

Vu and Kim[93] investigated HDC fed-batch culture of *Saccharomyces cerevisiae* KV-25 using molasses and corn steep liquor. The *Saccharomyces cerevisiae* KV-25 cell mass was significantly dependent on the ratio between C and N sources. In the fed-batch culture, the feed phase was preceded by a batch phase using the optimized medium, and a very high dried-cell-mass yield of 187.63 g L^{-1} was successfully attained by feeding a mixture of 20 % (v/v) molasses and 80 % (v/v) CSL at a rate of 22 mL h^{-1}. In this system, the production of cell mass depended mainly on the agitation speed, the composition of the feed medium, and the glucose level in the medium, but only slightly on the aeration rate.

Cellular stress

Even if the feeding profile of required substrates and oxygen supply are optimized, HDC results in cellular stress of the proliferated microorganisms. Singh *et al.*[94] investigated the cellular stress during different feeding rates in an *E. coli* fermentation process by transcriptome analysis. They found the production of heat shock proteins to be strongly upregulated. Simultaneously, the uptake of substrates was affected by the feeding rate and the followed growth rate (μ) by a regulation of the responsible gene expression. Also, the genes for the enzymes of the tricarboxylic acid cycle were strongly affected by the feeding rate. These insights in the metabolic functions are very helpful tools to overcome metabolic bottlenecks and improve feeding strategies for enhanced productivity and product quality during HDC processes.

Outlook

Even though HDCs have been developed and optimized over several years, there is still a strong need for further optimization and research in this field to help biotechnological products compete with mineral oil-based production processes, and to achieve a bio-based economy. New techniques as 3D-printing[95] may help to improve existing fermentation processes (immobilization) and develop new ones. Further research is required to overcome the existing problems associated with gradients of temperature, pH, and substrate concentration in large-scale bioreactors. New developments in fermentation technology may help to achieve additional knowledge about the microbial and biochemical processes.

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